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Disposable electrochemical aptasensor for gluten determination in food

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ABSTRACT

Reliable detection of decreasing amounts of gluten in food is the only way of ensuring the safety of all coeliac patients. Results obtained with the method of choice, immunochemical assays, are not entirely comparable and many of them are sandwich assays that cannot recognize hydrolyzed proteins. In this work, we propose a competitive electrochemical sensor based on a recently described aptamer targeting the gliadin immunodominant peptide 33-mer that triggers the coeliac disease. The sensing layer is built on the surface of a screen-printed carbon electrode (SPCE) by adsorption of streptavidin and subsequent peptide immobilization. A competition between the peptide and gluten proteins from samples for a defined concentration of biotinylated Gli 4 aptamer is established. The aptamer bound to the peptide on the surface is finally measured after enzyme labelling and chronoamperometric detection of an enzymatically obtained electrochemically active product. This method is able to detect as low as 0.113 $\mu g\, L^{-1}$ of gliadin, which corresponds to 380 $\mu g\, kg^{-1}$ of gluten in food, taking all dilutions and conversion factors into consideration, with a reproducibility lower than 11%. The aptasensor was applied to food samples with gluten contents above and below the legislated threshold for gluten-free labelling in the EU, obtaining good agreement with the official R5 immunochemical method.

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1. Introduction

Coeliac disease patients face the challenge to interpret a non-harmonized labelling in food across the world to avoid the offending compound that triggers their chronic autoimmune illness: gluten. This is in fact a mixture of storage proteins in wheat, barley, rye and possibly oat. The meaning of "gluten-free" label can vary from undetectable in Australia and New Zealand to 20 $mg\, kg^{-1}$ in the European Union [1]. The latter was set since the advent of immunochemical methods based on R5 antibody [2]. Unfortunately, the established level is still harmful for specially sensitized individuals. A proposal to decrease this value to 3 $mg\, kg^{-1}$ has been claimed [3] but the lack of more sensitive analytical methods along with the absence of a clinically proved maximum tolerated intake precludes its decrease so far.

Currently, there are two main groups of methods for gluten determination: those targeting directly the allergenic proteins or their peptide fragments, and those directed to identify DNA sequences specific of the carrier cereals. However, only a few of them have seen the transition to point of care by integration into chemical sensors.

Electrochemical genosensors have recently emerged as a novel low-cost strategy to detect gluten in food samples using a gene encoding the immunodominant peptide 33-mer as a target [4]. In combination with a structured capture probe the sensor was able to discriminate wheat from other gluten-containing cereals [5] unlike real-time PCR methods [6]. Though good correlation between immunochemical and genosensor results has been reported for a set of samples, DNA-based methods are indirect methods because they do not measure the offending compounds. They are excellent for identification and complementary to ELISA assays in samples where proteins are lacking or are highly degraded.

Proteomic-based methods are powerful tools for identification of immunotoxic peptides from different cereal sources but only the combination of HPLC with electrospray ionization (ESI) and tandem mass spectrometry detection (LC–MS/MS) allows quantitation at low $mg\, kg^{-1}$ level at expense of cumbersome and time-consuming extraction and digestion steps to avoid matrix effects [7]. The final peptide content, however, depends on the composition of the gluten sample and the yield of enzymatic digestion. It also requires

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a conversion into gluten units, a non-trivial challenge currently unavailable, to serve as surveillance method about the compliance with legislation. Therefore, this methodology is restricted to large research facilities and regarded as complementary to immunochemical assays in case of controversy [1].

The development of chemical sensors targeting the allergen requires a specific receptor, recognizing the proteins with high affinity. In the last decade, new antibodies have been raised against different epitopes present in prolamins, especially in gliadin, the wheat prolamin. These receptors are mainly used in the development of immunoasays. There are 36 different commercial ELISA kits in several formats: sandwich, competitive, lateral flow devices and dipsticks [1]. However, results obtained with them are not fully comparable even in simple samples. Not a single kit performs well in all matrices. The differences increase in challenging samples containing heated or hydrolyzed gluten or interference ingredients such salt, sugar or spices [8]. This indicates that there is a long way to reliably measure gluten in food samples using immunochemical approaches. Only two electrochemical immunosensors have been reported and used in real samples: a sandwich immunosensor that needs multiple steps to covalently attach the capture antibody to a self-assembled monolayer in addition to the binding and labeling steps and a 10-min enzymatic reaction before measurement [9]; and a competitive immunosensor with a limit of detection above 100 mg kg^{-1} due to incompatibility with extraction reagents [10].

At the research level, antibodies raised against deamidated gluten have been recently reported. This type of gluten is more toxic for coeliac patients and is profusely used in industry for several purposes in meat products, baked items and other food products. However, current antibodies fail to detect them, which compromise their safety. A lateral flow device with a limit of detection of 2 mg kg^{-1} in food can detect both native and deamidated gluten [11]. Superparamagnetic microsphere-based suspension array platforms are emerging as systems for multiplex analysis by flow cytometry. These beads contain different proportions of two dyes and each set is linked to a different antibody, which allows the simultaneously detection of up to 100 analytes. Quantification is based on a third reporter fluorophore. With these systems three allergens (casein, soy and gluten) were detected in a single assay in prepared mixtures [12]. Other platform under evaluation for commercialization can detect simultaneously 15 allergens including gluten [13].

Another possibility is to employ a glutamine-binding protein, which is able to bind glutamine residues in glutamine-rich peptides. This protein is successfully used to detect gluten-digested peptides with higher affinity than the intact gluten by fluorescence on a protein microarray. However, this strategy also relies on peptide detection similarly to MS proteomics [14].

Recently aptamers have emerged as novel receptors for gluten determination. Aptamers are short DNA or RNA oligonucleotides that can recognize a great variety of ligands with high specificity and selectivity. They are less prone to thermal denaturation and can be easily modified to include marker or anchoring functionalities. Aptamers were evolved against the immunodominant peptide known as 33-mer [15], the main peptide triggering the coeliac disease due to its resistance to digestion. Limits of detection as low as 0.5 mg kg^{-1} of gluten have been reported in competitive electrochemical aptamagnetoassays [16]. In spite of the easiness of handling and amenability to washing steps, magnetic microbeads are not fully accepted in routine agri-food labs yet. In response to industrial demands, we have developed an electrochemical competitive aptasensor. This approach showed a slightly improved detectability that rivals the most sensitive immunochemical assays and was successfully validated against a commercial ELISA assay in unknown food samples.

2. Materials and methods

2.1. Reagents

5′-Biotin tagged DNA aptamer (Gli 4) 5′-CCA GTC TCC CGT TTA CCG CGC CTA CAC ATG TCT GAA TGC C-3′ was obtained from Integrated DNA Technologies, IDT, Leuven, Belgium. Biotinylated 33-mer peptide was obtained from Biomedal, (Sevilla, Spain) with the following sequence: LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPFHHHHHH-lysine-biotin.

Gliadin standard solutions were prepared using the gliadin standard provided by the Working Group on Prolamin Analysis (PWG) and acquired to R-Biopharm. Streptavidin-peroxidase conjugate (Str-HRP_2) was obtained from Thermo Fisher (Madrid, Spain). Reagents for buffer preparation Tris:HCl pH 7.4, phosphate buffer saline (PBS, pH 7.4), NaCl, bovine serum albumin (BSA), $MgCl_2$, tween® 20 and ethylendiaminetetraacetic acid (EDTA),were DNAse and RNAse free (for molecular biology) and purchased from Sigma-Aldrich (Madrid, Spain). Streptavidin, biotin and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA were also acquired to Sigma. All solutions were prepared with MilliQ purified water.

2.2. Instrumentation

Electrochemical measurements were carried out with a μAutoLab type II potentiostat (Eco Chemie, The Netherland) using a three-electrode system. Screen-printed carbon electrodes from Dropsens (Oviedo, Spain) were used as working electrodes. External Ag|AgCl|KCl (sat) reference electrode and Pt counter electrode were used instead of the ones printed in the cell that were disassembled by covering with non-surfactant enamel. Both electrodes were inserted in a syringe filled with saturated KCl and stopped with filter paper as a liquid junction and placed just over the drop on the working electrode.

2.3. Procedures

2.3.1. Conditioning and preparation of the sensing layer on SPCE

Electrochemical screen-printed cells were washed with ethanol and MilliQ water and dried with a N_2 stream. Then the pseudoreference and counter electrodes were covered with non-surfactant enamel to precisely define the electroactive area avoiding non-specific adsorption.

All steps were performed in 0.1 M PBS. A 10 μL drop of 1 mg mL^{-1} streptavidin was placed onto the SPCE, incubated overnight at 4 °C and then washed with PBS with and without 0.001% tween® 20 (Fig. 1A, step 1). The electrode surface was blocked with 40 μL of 1% BSA for 30 min at room temperature and then washed as previously indicated (Fig. 1A, step 2). The biotinylated 33-mer peptide at 0.2 μM concentration was bound to the streptavidin layer for 1 h and then a washing step was also carried out (Fig. 1A, step 3). The unoccupied streptavidin binding sites were blocked with an excess of biotin (2 μM) for 30 min (Fig. 1A, step 4).

2.3.1.1. Binding curve. 10 μL of increasing concentrations of Gli 4 aptamer prepared in binding buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl and 5 mM $MgCl_2$) were incubated onto different modified electrode surfaces for 30 min at room temperature. Several washing steps with binding buffer with and without 0.001% tween® 20 to remove the weakly bound aptamers were carried out. The labelling step was performed by incubating 2.5 μg mL^{-1} Strep-HRP_2 in binding buffer containing tween® 20 for 30 min at room temperature. After several washing steps, 40 μL of TMB were added and the enzymatic reaction proceeded for 30 s. Then the cur-

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